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**SPECIFICATION**

**PROTEIN DRUG SUSTAINED-RELEASE MICROPARTICLE PREPARATION FOR  
INJECTION AND PROCESS FOR PRODUCING THE SAME**

5

Technical Field

[0001]

The present invention relates to protein drug  
10 sustained-release microparticle preparations for injection  
comprising, as a base, microparticles of a porous apatite or  
derivative thereof that slowly disappear in vivo, and to a  
process for producing the same.

15 Background Art

[0002]

Investigation has heretofore been made on protein drug  
sustained-release preparations for injection that slowly  
release the protein drugs over a long period, most of which  
20 comprise copoly(lactic-glycolic acid) (PLGA) as a base (see e.g.,  
Patent Documents 1 and 2 and Non-Patent Documents 1, 2, and  
3). Actually, sustained-release microcapsules that contain  
human growth hormone (hGH) and comprise PLGA as a base are  
in practical use in treatment in U.S. (see e.g., Non-Patent  
25 Document 4). PLGA is a biodegradable base that hydrolyzes  
and slowly disappears in a living body, and this property  
is preferable for a base of an injection. To produce

sustained-release preparations using PLGA, organic solvents for dissolving it are generally used. On the other hand, many protein drugs are water-soluble and are therefore used together with an organic solvent solution and an aqueous solution in order to produce their sustained-release microparticle preparations using PLGA.

[0003]

The simultaneous use of these two solvents leads to the denaturation and deactivation of some protein drugs. Such reduction in activity not only impairs efficacy but poses the risk of adversely affecting a living body. Water-soluble protein drug sustained-release microparticle preparations inevitably invite the transient excessive release of the protein drug in the early stage of administration (immediately after administration). Human growth hormone (protein drug) sustained-release microparticle preparations for injection using hydroxyapatite have also been reported (see e.g., Non-Patent Documents 5 and 6). However, all the preparations are two-component systems that have a particle size of apatite as large as 40 to 80  $\mu\text{m}$  or 200  $\mu\text{m}$  and are therefore difficult to administer by injection with a thin needle. Furthermore, their in vivo sustained-release effects are unknown. Besides, the amount of human growth hormone contained in apatite was as low as 1% or less.

[0004]

Patent Document 1: Japanese Patent Laid-Open No. 10-231252

Patent Document 2: U.S. Patent No. 5656297

Non-Patent Document 1: O.L. Johnson et al: Nature Medicine,  
2: 795-799, (1996)

Non-Patent Document 2: M. Takenaga et al: J. Pharmacy  
5 Pharmacology, 54: 1189-1194, (2002)

Non-Patent Document 3: S. Takada et al: J. Controlled  
Release, 88: 229-242, (2003)

Non-Patent Document 4: NDA 21-075

Non-Patent Document 5: J. Guicheux et al: J. Biomedical  
10 Materials Research, 34: 165-170, (1997)

Non-Patent Document 6: H. Gautier et al: J. Biomedical  
Materials Research, 40: 606-613, (1998)

#### Disclosure of the Invention

15 [0005]

For protein drug sustained-release preparations for  
injection, there are many challenges to produce the  
preparations: materials having in vivo disappearing  
capabilities, which disappear from living bodies toward the  
20 end of drug release after administration, must be selected;  
in the production thereof, the preparations must avoid the  
simultaneous use of an organic solvent immiscible with water  
and an aqueous solution and circumvent the denaturation of  
the protein drugs; moreover, a drug content in the  
25 microparticle preparation must be at least 5% or more,  
otherwise it is difficult to administer with a thin needle  
due to their too large doses; the preparations must be able

to pass through a thin needle because they are repetitively administered in many cases; and the microparticle preparations must slowly release the contained protein drug over a period of at least three days or more, preferably one week or more; and must minimize initial burst release likely to cause side effects.

[0006]

Thus, an object of the present invention is to provide protein drug sustained-release microparticle preparations for injection that in the production thereof, minimize the use of organic solvents and avoid the simultaneous use of an organic solvent immiscible with water and an aqueous solution and that with respect to the resulting product, have both bioerodibilities and sustained-release capabilities, slowly release the contained protein drug at a substantially constant rate over a period of three days or more and realize a drug content of 5% or more, excelling in dispersibility and needle passability, and to provide a process for producing the same.

[0007]

To solve these challenges, the present inventors have found that preparations that have both bioerodibilities and sustained-release capabilities are obtained by utilizing microparticles of a porous apatite or derivative thereof without the simultaneous use of water and an organic solvent. The present inventors have further found that the initial burst release of a protein drug is suppressed by utilizing the protein drug in combination with a water-soluble divalent metal

compound. In addition, the present inventors have also found that sustained-release capabilities over a longer period and smaller initial burst release are simultaneously attained by sufficiently infiltrating a protein drug into a porous apatite and providing an in vivo disappearing polymer compound thereon by coating or adhesion.

[0008]

The porous apatite and derivative thereof constituting the protein drug sustained-release microparticle preparation for injection described herein may be hydroxyapatite or a compound in which a portion of calcium as a component thereof is substituted with zinc. In this context, the rate of zinc substitution is preferably 1 to 20%. Microparticles of the porous apatite and derivative thereof can be obtained by a known method. Examples of the method include the method described in T. Yamaguchi, H. Yanagida (eds.), A. Makishima, H. Aoki, Ceramic Science Series 7: Bioceramics, GIHODO SHUPPAN Co., Ltd., pp. 7-9, 1984. In vivo disappearing speed differs depending on the ratio of calcium (Ca) and phosphorus (P) constituting hydroxyapatite. If a value of  $(Ca+Zn)/P$  is smaller than 1.67, higher water-solubility and higher in vivo disappearing speed are attained. It is preferred that the value of  $(Ca+Zn)/P$  should fall within the range of 1.67 to 1.51. A lower treatment temperature at which the apatite is produced renders water solubility higher and disappearing speed higher. The treatment temperature used is generally room temperature to 800°C, preferably 150°C to 600°C, more

preferably 150°C to 400°C. If the apatite is burned at 800°C or higher, it does not disappear in vivo. If the apatite is treated at 100°C or lower, particles thereof tend to agglomerate together and are therefore difficult to administer by injection with an ordinary needle. The apatite is preferably used at a particle size of 50  $\mu\text{m}$  or lower in average. However, if the particle size is too small, the encapsulation rate of the protein drug might be decreased. Therefore, the particle size used is preferably 0.1 to 50  $\mu\text{m}$ , more preferably 0.5 to 30  $\mu\text{m}$ , even more preferably 0.5 to 10  $\mu\text{m}$ .

[0009]

The in vivo disappearing polymer compound for coating this porous apatite includes polylactic acid (PLA) or copolylactic-glycolic-acid (PLGA), a block copolymer comprising PLA and/or PLGA bound with polyethyleneglycol (PEG), collagen, polycyanoacrylic acid, and polyamino acid derivatives. PLA or PLGA used at a high concentration allows apatite particles coated with the in vivo disappearing polymer to agglomerate together. Since an organic solvent immiscible with water is used in this procedure, the protein drug might be denatured due to water added in freeze drying at the final step unless this organic solvent is completely removed. From studies in various ways, it has been concluded that the block copolymer comprising PLA or PLGA bound with PEG is preferable. This block copolymer may be a compound with a binding style in which PLA or PLGA is bound through ester bond with hydroxyl groups at both ends of PEG or bound through ester bond with

hydroxyl group at one end of PEG. For the ester bond with one end of PEG, it is preferred that the other end should be protected with  $\text{OCH}_3$ , an alkoxy group, or the like.

Alternatively, it may be bound with a functional group such as amino and carboxyl groups. Concerning the ratio of PEG and PLA or PLGA, the block copolymer has preferably 20 to 90% by weight, more preferably 25 to 65% by weight, of PEG. The molecular weight of the block copolymer is preferably 3,000 to 20,000, more preferably 5,000 to 12,000, in its entirety.

10 The amount of the biodegradable block copolymer used is in the range of generally 3 to 100% by weight, preferably 5 to 30%, with respect to that of the apatite derivative.

[0010]

The water-soluble divalent metal compound includes zinc chloride, zinc acetate, zinc carbonate, calcium chloride, calcium hydroxide, ferrous or ferric chloride, ferrous or ferric hydroxide, and cobaltous or cobaltic chloride. The zinc chloride is particularly preferably used. The zinc chloride may be used in combination with sodium carbonate or sodium bicarbonate. The amount of the water-soluble divalent metal compound used differs depending on the encapsulated protein drug and is in the range of generally preferably 2 to 100% by weight, more preferably 2 to 30% by weight, with respect to that of the porous apatite.

15

20

25 [0011]

The protein drug is defined as a compound having a molecular weight of 5,000 or more. Examples thereof include

human growth hormone, hepatocyte growth factor (HGF),  
fibroblast growth factor (FGF), IGF-1, EGF, NK4, VEGF, NGF,  
BDNF, BMP, adiponectin, interferons (INF- $\alpha$ ), interleukins  
(such as IL-2, IL-4, and IL-5), EPO, G-CSF, insulin, ANP, TNF- $\alpha$ ,  
5 and antibodies. The amount of the protein drug used differs  
depending on the protein drug activity and a sustained-release  
period. A larger amount of the protein drug encapsulated in  
the porous apatite is more preferable. The amount of the  
protein drug stably encapsulated in the porous apatite is  
10 generally 5 to 50% with respect to that of the apatite.  
[0012]

A process for producing the protein drug  
sustained-release microparticle preparation for injection of  
the present invention is generally performed by procedures  
15 as described below. Microparticles of a porous apatite or  
derivative thereof are dispersed in an aqueous solution of  
a protein drug, and the dispersion is stirred to sufficiently  
infiltrate the aqueous solution into the apatite. Then, the  
aqueous solution that could not be infiltrated therein is  
20 removed by centrifugation and so on. If necessary, an aqueous  
solution of a water-soluble divalent metal compound is further  
added thereto and stirred to infiltrate the aqueous solution  
thereinto. Subsequent filtration and vacuum drying or freeze  
drying give a powder containing the protein drug. This powder  
25 is dispersed in an aqueous solution or suspension of an  
biodegradable block copolymer or in an aqueous solution or  
suspension of a biodegradable block copolymer containing a



solvent miscible with water (e.g., acetone and ethanol), the dispersion is stirred and, if necessary, supplemented with a stabilizer or the like, followed by freeze drying or vacuum drying to produce the preparation in a powder form. This powder, when actually administered, is dispersed in an appropriate dispersion medium and subcutaneously or intramuscularly administered by injection. The particle size of the finally obtained sustained-release microparticle preparation may be a size that allows the preparation to pass through an injection needle used in typical administration. In reality, the smaller size an injection needle has, the less a patient is scared. It is more preferred that the preparation should pass through an injection needle with a thickness of 25G or smaller defined by the international standard that specifies the thickness of an injection needle. The particle size of the sustained-release microparticle preparation that satisfies these requirements is 0.5 to 50  $\mu\text{m}$ . Moreover, the sustained-release period of the protein drug differs depending on drug activity and so on and is generally preferably one week or more.

[0013]

It was found that microparticle preparations obtained by the present invention slowly release the contained protein drug over a period of at least three days or more and realize quite small initial burst release and a protein drug content reaching even 30% at maximum. Moreover, the resulting preparations passed through a 25G injection needle.

Furthermore, the microparticle preparations can be prepared finally into a powdered form by freeze drying, and the encapsulated protein drug is very stable.

## 5 Examples

[0014]

Hereinafter, the sustained-release and in vivo disappearing capabilities of preparations of the present invention will be illustrated with reference to Examples.

10 However, the present invention is not intended to be limited to these Examples.

[0015]

(Example 1)

Two types of hydroxyapatites with zinc substitution  
15 (average particle size: 8  $\mu$ m) burned at different temperatures were used to conduct a confirmation test on in vivo disappearance. Five SD male rats (6 weeks old) per group were used. The product treated at 180°C and the product treated at 400°C were separately dispersed in suspensions (0.1% Tween  
20 80, 0.5% CMC-Na, and 5% mannitol aqueous solution), and 5 mg each of the products was bilaterally administered at a dose of 0.2 ml/rat to the middle part of the dorsal hypodermis. Residuals in the administration sites were excised periodically (on 3 hours, 1, 5, 10, 15, and 20 days) after  
25 administration. The wet weights and calcium levels thereof were measured (Tables 1 and 2). The wet weight were nearly doubled due to swelling and so on from 1 to 5 days after

administration, while the calcium levels were slightly increased. Thereafter, the disappearance of both the wet weight and the calcium levels rapidly proceeded. They almost disappeared on around 15 days for the product treated at 180°C and on 20 days for the product treated at 400°C.

[0016]

Table 1 Wet weights of extracted hydroxyapatite (mg)

	3 hours	1 day	5 days	10 days	15 days	20 days
Product treated at 180°C (n=5)	14.2	27.1	28.0	17.0	2.3	0.0
Product treated at 400°C (n=5)	14.0	23.9	29.1	23.2	10.8	0.0

[0017]

Table 2 Residual calcium levels of excised hydroxyapatite (mg)

	3 hours	1 day	5 days	10 days	15 days	20 days
Product treated at 180°C (n=5)	1.27	1.53	1.45	0.26	0.01	0.00
Product treated at 400°C (n=5)	1.33	1.62	1.91	0.48	0.20	0.00

[0018]

(Example 2)

A derivative (HAp-Zn-0.5 (100 mg): 5% of calcium in hydroxyapatite (HAp) was substituted with zinc; 0.5 mol zinc with respect to 9.5 mol calcium) burned at 400°C was supplemented with 700  $\mu$ L of hGH solution (50 mg/mL) desalted with a PD-10 column (Amersham Pharmacia) and then with water

to bring the final amount of the solution to 5 mL. After stirring for 3 minutes, the solution was centrifuged at 3,000 rpm for 3 minutes. The resulting pellet was supplemented with 10 mL of water and stirred for 1 minute. The suspension was  
5 centrifuged again at 3,000 rpm for 3 minutes. The resulting pellet was supplemented with 2.0 mL of aqueous solution of 20.4 mg/mL zinc chloride (300  $\mu$ mol zinc chloride; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and stirred with a touch mixer, followed by freeze drying. PLA-PEG-PLA-Y004  
10 (PEG ratio: 32%; molecular weight: 8,200) was dissolved at a concentration of 20% in acetone. This acetone solution was mixed with water at a 1:4 ratio to produce an acetone-water mixture solution containing the the block copolymer. The resulting freeze-dried powder was supplemented with 500  $\mu$ L  
15 of this acetone-water mixture solution containing the polymer and well stirred with a touch mixer, followed by freeze drying. A preparation untreated with polymer solution was produced as a control. A hGH content in the resulting preparations was quantified with micro BCA protein assay kit (Pierce).  
20 [0019]

The in vitro release capabilities of the resulting hGH microparticle preparation samples were compared. The precisely weighed 2.5 mg aliquot of each of the resulting hGH microparticle preparation samples was supplemented with 0.250  
25 mL of PBS (phosphate buffered saline) and stirred at 37°C. The supernatant was collected periodically by centrifugation at 3000 rpm for 3 min. The amount of hGH released into the

supernatant was quantified by gel filtration HPLC analysis (TOSO G2000SW-x1). This result is shown in Table 3. The release of hGH into PBS was suppressed more in the preparation treated with polymer solution than in the preparation untreated with polymer solution produced as a control.

[0020]

Table 3 Influence of polymer solution treatment on in vitro release capabilities of hGH microparticle preparation

Polymer solution treatment	Cumulative amount of hGH released ( $\mu$ g)				
	2 hr	4 hr	24 hr	4 day	7 day
Untreated (n=2)	0.8	2.3	6.3	7.8	9.6
Treated (n=2)	1.5	2.6	2.6	2.6	2.9

[0021]

(Example 3)

HAp-Zn-0.5 (100 mg) treated at 400°C was supplemented with 700  $\mu$ L of hGH solution (50 mg/mL) desalted with a PD-10 column (Amersham Pharmacia) and then stirred for 1 minute with a touch mixer. Next, the solution was supplemented with water to bring the final amount of the solution to 5 mL, followed by stirring for 1 minute with a touch mixer. The suspension was left undisturbed for 3 minutes and centrifuged at 3,000 rpm for 3 minutes. The resulting pellet was supplemented with 5 mL of water and stirred again for 1 minute. The suspension was centrifuged again at 3,000 rpm for 3 minutes. The resulting pellet was supplemented with 2.7 mL of aqueous solution of 20.4 mg/mL zinc chloride (400  $\mu$ mol zinc chloride; Wako Pure

Chemical Industries, Ltd., Osaka, Japan) and stirred with a touch mixer, followed by freeze drying.

PLA-PEG-PLA-Y001 (PEG ratio: 65.4%; molecular weight: 14,600) was dissolved at a concentration of 20% in acetone. 5 This acetone solution was mixed with water at a 1:4 ratio to produce an acetone-water mixture solution containing the polymer. The resulting freeze-dried powder was supplemented with 500  $\mu$ L of this acetone-water mixture solution containing the polymer and well stirred with a touch mixer, followed by 10 freeze drying. A preparation untreated with polymer solution was produced as a control. A hGH content in the resulting hGH microparticle preparations was quantified with micro BCA protein assay kit (Pierce).

[0022]

15 Each of the produced hGH microparticle preparations was suspended in 0.5% CMC-Na, 5% mannitol, and 0.1% Tween 80 and subcutaneously administered at 10 IU/kg (1 IU: 0.35 mg) to the dorsal site of a male SD rat.

Blood was collected from the tail vein at 1, 2, 4, and 20 8 hours after administration and subsequently on the daily basis to measure a hGH concentration in blood with an automatic EIA apparatus AIA-6000 (Tosoh) and E Test "TOSOH" II (hGH). This result is shown in Table 4. The higher hGH concentration in blood was maintained for a longer time in the preparation 25 treated with polymer solution than in the preparation untreated with polymer solution.

[0023]

Table 4 In vivo sustained-release effect of hGH microparticle preparation

Polymer solution treatment	hGH content (%)	hGH concentration in blood (ng/mL)							
		4 hr	8 hr	1 day	2 day	3 day	4 day	5 day	6 day
Untreated (n=2)	16.2	26.4	49.1	11.8	2.8	1.3	0.78	0.43	0.32
Treated (n=2)	11.2	42.6	65.4	21.6	9.7	4.4	2.2	1.4	0.49

5 [0024]

(Example 4)

HAp-Zn-0.5 (100 mg) treated at 400°C was supplemented with 700 µL of hGH solution (50 mg/mL) desalted with a PD-10 column (Amersham Pharmacia) and subsequently with water to  
10 bring the final amount of the solution to 5 mL. After stirring for 3 minutes, the solution was centrifuged at 3,000 rpm for 3 minutes. The resulting pellet was supplemented with 10 mL of water and stirred for 1 minute. The solution was centrifuged again at 3,000 rpm for 3 minutes. The resulting pellet was  
15 supplemented with 2.0 mL of aqueous solution of 20.4 mg/mL zinc chloride (300 µmol zinc chloride; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and stirred with a touch mixer, followed by freeze drying.

PLA-PEG-PLA-Y004 (PEG ratio: 32%; molecular weight:  
20 8,200) was dissolved at a concentration of 20% in acetone. This acetone solution was mixed with water at a 1:4 ratio to produce an acetone-water mixture solution containing the polymer. The resulting freeze-dried powder was supplemented

with 500  $\mu$ L of this acetone-water mixture solution containing the polymer and well stirred with a touch mixer, followed by freeze drying. A hGH content in the resulting hGH microparticle preparation was quantified with micro BCA protein assay kit (Pierce).

[0025]

The produced hGH microparticle preparation was suspended in 0.5% CMC-Na, 5% mannitol, and 0.1% Tween 80 and subcutaneously administered at 30 IU/kg (1 IU: 0.35 mg) to the dorsal site of a male SD rat immunosuppressed with tacrolimus (Fujisawa Pharmaceutical Co., LTD., Osaka, Japan). The tacrolimus was administered in advance at a dose of 0.4 mg/rat on 3 days before the administration of the preparation and subsequently at a dose of 0.2 mg/rat at 3-day intervals after the initiation of subcutaneous administration of the preparation to the dorsal site.

Blood was collected from the tail vein at 1, 2, 4, and 8 hours after the administration of the preparation and subsequently every one or two days to measure a hGH concentration in blood with an automatic EIA apparatus AIA-6000 (Tosoh) and E Test "TOSOH" II (hGH). This result is shown in Table 5. The sustained-release effect over a period of approximately 2 weeks was observed in the hGH microparticle preparation treated with polymer solution.

[0026]

Table 5 In vivo sustained-release effect of hGH microparticle preparation (hGH content: 14.3%)



Time elapsed after administration	4 hr	8 hr	12 hr	16 hr	1 day	2 day	3 day	4 day	5 day
hGH concentration in blood (ng/mL)	4.6	31.6	99.9	101.3	95.7	29.9	11.4	8.5	5.8

6 day	7 day	8 day	9 day	10 day	11 day	12 day	14 day
4.2	3.4	2.6	2.0	1.8	1.6	1.9	1.3

[0027]

(Example 5)

A derivative (HAp-Zn-0.5 (150 mg): a portion of calcium  
 5 in HAp was substituted with zinc; 0.5 mol zinc with respect  
 to 9.5 mol calcium) burned at 400°C was supplemented with 525  
 µL of interferon-α (IFN-α) solution (2.86 mg/mL) and then with  
 water to bring the final amount of the solution to 2 mL. After  
 stirring for 5 minutes, the suspension was centrifuged at 3,000  
 10 rpm for 3 minutes. The resulting pellet was supplemented with  
 15 mL of water and stirred for 1 minute. The suspension was  
 centrifuged again at 3,000 rpm for 3 minutes. The resulting  
 pellet was supplemented with 2.0 mL of aqueous solution of  
 20.4 mg/mL zinc chloride (300 µmol zinc chloride; Wako Pure  
 15 Chemical Industries, Ltd., Osaka, Japan) and stirred with a  
 touch mixer, followed by freeze drying. PLA-PEG-PLA (PEG  
 ratio: 32%; molecular weight: 8,200) was dissolved at a  
 concentration of 20% in acetone. This acetone solution was  
 mixed with water at a 1:4 ratio to produce an acetone-water  
 20 mixture solution containing the polymer. The resulting  
 freeze-dried powder was supplemented with 750 µL of this

acetone-water mixture solution containing the polymer and well stirred with a touch mixer, followed by freeze drying. A preparation free of polymer solution treatment was produced as a control. An IFN- $\alpha$  content in the resulting HAp-IFN- $\alpha$  preparations was quantified with Human Interferon Alpha (Hu IFN- $\alpha$ ) ELISA Kit (PBL Biomedical Laboratories).  
[0028]

The in vitro release capabilities of the resulting HAp-IFN- $\alpha$  samples were compared. The precisely weighed 2.5 mg aliquot of each of the resulting HAp-IFN- $\alpha$  samples was supplemented with 0.25 mL of 1/10 PBS (phosphate buffered saline) and stirred at 37°C. The supernatant was collected periodically by centrifugation at 3000 rpm for 3 minutes. The amount of IFN- $\alpha$  released into the supernatant was quantified with Human Interferon Alpha (Hu IFN- $\alpha$ ) ELISA Kit (PBL Biomedical Laboratories). This result is shown in Table 6. The release of IFN- $\alpha$  into 1/10 PBS was suppressed more sufficiently in the preparation treated with polymer solution than in the preparation untreated with polymer solution produced as a control.  
[0029]

Table 6 Influence of polymer solution treatment on in vitro release capabilities of HAp-IFN- $\alpha$  preparation

	Cumulative amount of interferon- $\alpha$ released (pg)						
	0	1 hr	2 hr	4 hr	24 hr	4 day	7 day
Untreated with polymer solution	455	989	2601	3398	4017	4727	6338

Treated with polymer solution	95	207	328	410	550	641	752
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[0030]

(Example 6)

HAp-Zn-0.5 (100 mg) treated at 400°C was supplemented  
5 with 3.5 mL of solution of a recombinant human insulin (Wako  
Pure Chemical Industries, Ltd., Osaka, Japan) dissolved at  
10 mg/mL in 0.01 N HCl and further with 0.01 N HCl to bring  
the final amount of the solution to 5 mL. After stirring for  
3 minutes, the suspension was centrifuged at 3,000 rpm for  
10 3 minutes. The resulting pellet was supplemented with 10 mL  
of water and stirred for 1 minute. The suspension was  
centrifuged again at 3,000 rpm for 3 minutes. The resulting  
pellet was freeze-dried. PLA-PEG-PLA-Y004 (PEG ratio: 32%;  
molecular weight: 8,200) was dissolved at a concentration of  
15 20% in acetone. This acetone solution was mixed with water  
at a 1:4 ratio to produce an acetone-water mixture solution  
containing the polymer. The resulting freeze-dried powder  
was supplemented with 500 µL of this acetone-water mixture  
solution containing the polymer and well stirred with a touch  
20 mixer, followed by freeze drying. A preparation untreated  
with polymer solution was produced as a control. A hGH content  
in the resulting preparations was quantified with micro BCA  
protein assay kit (Pierce).

[0031]

25 The invitro release capabilities of the resulting insulin  
microparticle preparation samples were compared with the

control. The precisely weighed 2.5 mg aliquot of each of the resulting insulin microparticle preparation samples was supplemented with 0.25 mL of PBS (phosphate buffered saline) and stirred at 37°C. The supernatant was collected  
5 periodically by centrifugation at 3000 rpm for 3 minutes. The amount of insulin released into the supernatant was quantified with micro BCA protein assay kit (Pierce) to calculate its rate with respect to the total amount of insulin contained in each preparation. This result is shown in Table 7. The  
10 release of insulin into PBS was suppressed more in the preparation treated with polymer solution than in the preparation untreated with polymer solution produced as a control.

[0032]

15 Table 7 Influence of polymer solution treatment on in vitro release capabilities of insulin microparticle preparation

Polymer solution treatment	Cumulative amount of insulin released (% of total insulin)				
	1 hr	2 hr	4 hr	24 hr	4 day
Untreated (n=2)	29.3	47.4	56.8	61.6	61.6
Treated (n=2)	21.8	39.8	49.0	49.0	49.0